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THE ISOPROSTANES: UNIQUE PROSTAGLANDIN-LIKE PRODUCTS OF FREE-RADICAL-INITIATED LIPID PEROXIDATION*

JASON D. MORROW,† YAN CHEN, CYNTHIA J. BRAME, JAMES YANG, STEPHANIE C. SANCHEZ, JEFFREY XU, WILLIAM E. ZACKERT, JOSEPH A. AWAD, and L. JACKSON ROBERTS
Department of Medicine and Pharmacology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232-6602

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* This paper was refereed by Victor M. Samokyszyn, Ph.D., Division of Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR 72205.

† To whom correspondence and reprint requests should be sent at 506 MRB-1, Vanderbilt University, 23rd and Pierce Avenues, Nashville, TN 37232-6602. Fax: (615) 322-4707; E-mail: jason.morrow@mcmail.vanderbilt.edu

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I. INTRODUCTION

Free radicals, largely derived from oxygen, have been implicated in the pathophysiology of many human diseases, including cancer, atherosclerosis, neurodegenerative disorders, and even the normal aging process [1–4]. Definitive evidence for this association is often lacking, however, because of recognized shortcomings with methods to assess oxidative stress status in vivo [5]

A well-recognized result of oxidant injury is peroxidation of lipids. In the early 1990s, we reported that a series of prostaglandin (PG)-like compounds are produced by the free-radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme, which had heretofore been considered obligatory for endogenous prostanoid synthesis [6]. Since then, we have accumulated a large body of evidence indicating that quantification of these unique products of lipid peroxidation, now termed isoprostanes (IsoPs), provides a reliable marker of oxidant injury both in vitro and in vivo. Furthermore, several of these compounds possess potent biological activity and, thus, may be mediators of oxidant injury. It is the purpose herein to summarize selected aspects of our knowledge regarding the IsoPs. This review will (1) highlight mechanisms involved in IsoP formation, (2) summarize the identification of a new class of IsoPs, termed A_2/J_2-

IsoPs, (3) review methods to analyze IsoPs, and (4) examine the utility of quantifying IsoPs in animal models of oxidant stress and as markers of oxidant injury in association with human disease.

II. HISTORICAL BACKGROUND

In the 1960s and 1970s, it was shown that PG-like compounds could be formed by the autoxidation of purified polyunsaturated fatty acids [7–10]. Elegant studies carried out by Pryor, Porter, and others led to a proposed mechanism by which these compounds were generated via bicycloendoperoxide intermediates [8]. However, this work was never carried beyond in vitro studies nor was it determined whether PG-like compounds could be formed in biological fluids containing unsaturated fatty acids.

Previously, we had shown that PGD_2 derived from the cyclooxygenase is primarily metabolized in vivo in humans to form 9α,11β-PGF_2 by the enzyme 11-ketoreductase [11]. In aqueous solutions, however, PGD_2 in an unstable compound that undergoes isomerization of the lower side chain and these isomers can likewise be reduced by 11-ketoreductase to yield isomers of 9α,11β-PGF_2 [12]. In studies undertaken to further characterize these compounds utilizing a gas chromatographic (GC)/mass spectrometric (MS) assay, we found that in plasma samples from normal volunteers that were processed and analyzed immediately, a series of peaks were detected possessing characteristics of F-ring PGs (Fig. 1). Interestingly, however, when plasma samples that had been stored at −20°C for several months were reanalyzed, identical chromatographic peaks were detected, but levels of putative PGF_2-like compounds were up to 100-fold higher [6]. Subsequent studies led to the conclusion that these PGF_2-like compounds were generated in both freshly processed and stored plasma, not by a cyclooxygenase-derived mechanism but nonenzymatically by autoxidation of plasma arachidonic acid [6,13]. Because these compounds contain F-type prostane rings, they are heretofore referred to as F_2-isoprostanes (F_2-IsoPs).

III. MECHANISM OF FORMATION OF THE ISOPROSTANES

A mechanism to explain the formation of the F_2-IsoPs is outlined in Fig. 2 and is based on that proposed by Pryor and colleagues for the generation of bicycloendoperoxide intermediates resulting from the peroxidation of other poly-

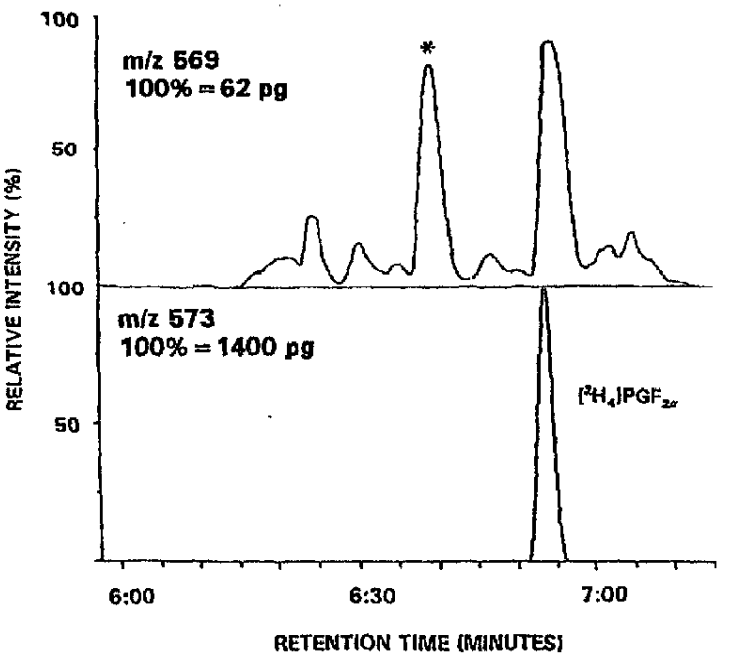

FIG. 1. Analysis of F_2-IsoPs in a plasma sample from a normal human volunteer. The m/z 569 ion current chromatogram represents endogenous F_2-IsoPs. The m/z 573 chromatogram represents the $[^2H_4]PGF_{2\alpha}$ internal standard. The peak represented by the asterisk (*) is the one routinely quantified for F_2-IsoPs. The concentration of F_2-IsoPs in this plasma sample was 58 pg/mL.

unsaturated fatty acids [8]. Precursor arachidonic acid shown at the top of Fig. 2 initially undergoes abstraction of an allylic hydrogen atom to yield an arachidonyl carbon-centered radical. Subsequently, there is coupling of dioxygen to yield peroxyl radicals. Depending on the site of hydrogen abstraction and oxygen insertion, four different peroxyl radical isomers are formed. Endocyclization of the radicals occurs, followed by the addition of another molecule of oxygen to yield four bicycloendoperoxide (PGG_2-like) regioisomers. These intermediates are then reduced to F_2-IsoPs. Each of the four regioisomers can theoretically be comprised of eight racemic diastereomers. Thus, a total of 64 different compounds

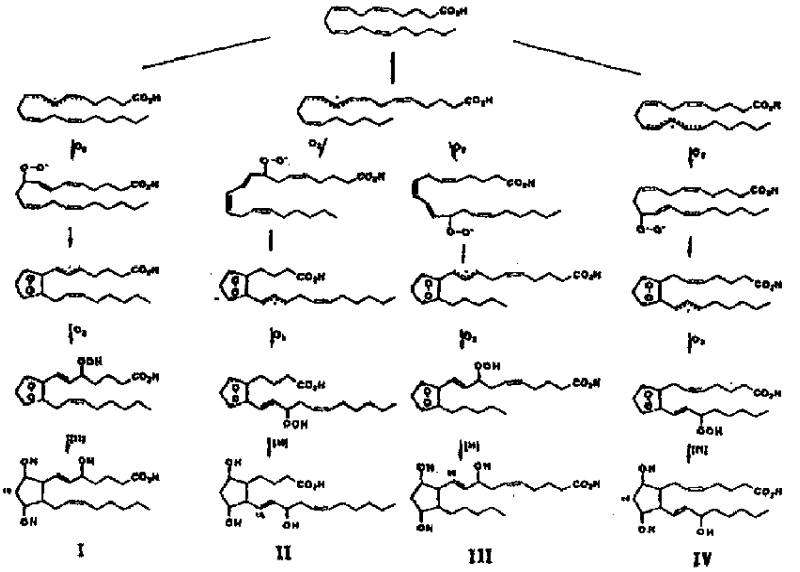

FIG. 2. Mechanism of formation of the F_2-IsoPs. This pathway leads to the formation of four regioisomers (I–IV). For simplicity, stereochemical orientation is not indicated. Each regioisomer theoretically is comprised of a mixture of eight racemic diastereomers. (Reprinted from Ref. 6 with permission from Academic Press.)

can be generated by this process; although, as discussed later, the formation of some is favored over others. Recently, in support of the proposed mechanism of formation, we have obtained direct evidence both in vitro and in vivo that each of the four classes of regioisomers are formed [14]. In addition, as might be expected, compounds comprising regioisomers I and IV predominate owing to the fact that regioisomers II and III derive from the same arachidonyl radical precursor.

Several structural aspects of the F_2-IsoPs should be noted in comparison to cyclooxygenase-derived PGs. Because F-ring compounds derive from the reduction of endoperoxide intermediates, the hydroxyls on the prostane ring must be oriented cis, although they can be α,α or β,β [6,10]. In addition, unlike cyclooxygenase-derived PGs, nonenzymatic generation of the IsoPs favors compounds in

which the side chains are predominantly oriented cis in relation to the prostane ring.

IV. FORMATION OF ISOPROSTANES IN VIVO

As noted, we initially discovered IsoPs as products of the oxidation of plasma arachidonic acid that had been stored at −20°C. Impressed that these compounds are readily formed in vitro, we sought to determine whether they might also be generated in vivo. Several observations suggested that this might be so. First, as mentioned, we were able to detect measurable quantities of F_2-IsoPs in fresh human plasma from normal volunteers analyzed immediately at levels of 35 ± 6 pg/mL (n = 12) [13,15]. However, because large quantities of IsoPs can be generated ex vivo, we were concerned whether these amounts represented true endogenous levels or whether they were formed ex vivo by autoxidation of plasma lipids. This latter possibility seemed unlikely for the following reasons. First, plasma contains significant quantities of antioxidants and it has been reported that lipid peroxidation is inhibited until endogenous ascorbate is virtually entirely consumed [16,17]. Second, we found that drawing blood into syringes containing the antioxidant butylated hydroxytoluene (BHT) or the reducing substance triphenylphosphine failed to reduce measured levels [13,18]. Third, we found that levels of F_2-IsoPs in urine from normal human volunteers were high (1.6 ± 0.6 ng/mg creatinine) [18]. Urine contains only minute amounts of lipids and, thus, it was unlikely that such substantial levels of these compounds would be generated ex vivo. Further support for this was the finding that urinary IsoP levels did not increase when urine was incubated at 37°C for up to 5 days [13,18]. Definitive evidence that IsoPs are formed in vivo was demonstrated by showing that levels of compounds detected in the plasma of rats treated with either CCl_4 or the herbicide diquat to induce an oxidant injury were increased up to 200 times the levels measured in control rats [13,19].

A second aspect related to the formation of isoprostanes is that they are formed in situ esterified to phospholipids in vivo. Only small amounts of arachidonic acid are present in the unesterified state and the vast majority exists esterified to phospholipids [20]. Thus, we examined whether F_2-IsoPs are initially formed esterified to phospholipids and are subsequently released in the free form by phospholipases. This was important because it counters the accepted dogma that prostanoids do not exist esterified in phospholipids. To support the hypothesis that IsoPs are initially formed in situ on phospholipids, we examined the time course of appearance of increases in levels of F_2-IsoPs esterified in liver phospholipids and free in the circulation, following administration of CCl_4 to rats to induce an oxidant injury [19]. Levels of esterified IsoPs increased rapidly, reaching

half-maximum concentrations in the liver within 15 min, whereas the appearance of increases in the circulation was delayed significantly (up to several hours) [19]. Direct evidence for the formation of F_2-IsoPs esterified to phospholipids was obtained when a lipid extract of liver tissue from rats treated with CCl_4 was subjected to high-performance liquid chromatographic (HPLC) purification using a straight-phase system that separates phosphatidylcholine from less polar lipids [20]. Fractions collected were then subjected to chemical hydrolysis and analyzed for free F_2-IsoPs to detect those that contained esterified F_2-IsoPs. We found that fractions containing presumed esterified F_2-IsoPs eluted in a region that was more polar than unoxidized phosphatidylcholine [20]. Analysis of these fractions by fast-atom-bombardment MS definitely identified phosphatidylcholine species with palmitate or stearate esterified at the sn-1 position and an F_2-IsoP at the sn-2 position. More detailed analyses of phospholipid-containing F_2-IsoPs have since been carried out utilizing collision-induced dissociation tandem MS [21].

As noted, after the administration of CCl_4 to rats, increased concentrations of F_2-IsoPs esterified in liver tissue can be detected, followed by increased levels in the circulation [19]. This suggests that free compounds derive, at least in part, from the hydrolysis of IsoPs from phospholipids in vivo. It is reasonable to assume that the hydrolysis is catalyzed by phospholipases. In vitro, we have found that bee (*Apis mellifera*) venom phospholipase A_2 efficiently hydrolyzes IsoPs from lipids [20], although the phospholipase(s) responsible for the hydrolysis of IsoPs in vivo remains to be established.

After determining that IsoPs are initially formed by peroxidation of arachidonic acid esterified to tissue lipids, we have analyzed a variety of normal animal tissues for levels of esterified F_2-IsoPs, including liver, testes, heart, brain, skeletal muscle, aorta tissue, ocular lens, kidney, and lung, and found detectable levels in all of these tissues [18]. Analysis of human tissues has been limited to gastric biopsies, where levels of F_2-IsoPs in the nanogram per gram tissue are present. In addition, F_2-IsoPs are detectable in human cerebrospinal fluid at picogram per milliliter concentrations and are significantly increased in patients with Alzheimer's disease, a chronic neurodegenerative disorder associated with increased oxidant stress in the central nervous system (unpublished data). These findings are consistent with the fact that detectable levels of unesterified F_2-IsoPs are present in all normal biological fluids from both animals and humans that have been tested to date.

A. D_2/E_2-Isoprostanes

The cyclooxygenase-derived endoperoxide intermediate, PGH_2, is unstable and rearranges in aqueous solutions to PGD_2 and PGE_2 with a half-life of several minutes [22]. Analogously, it was reasonable to suspect that the intermediate

IsoP endoperoxides, if not efficiently reduced to F_2-IsoPs, may also rearrange to form D- and E-ring IsoPs. Experiments were carried out to explore this possibility, which convincingly demonstrated that D_2/E_2-IsoPs are also formed in vivo [23]. Levels of D_2/E_2-IsoPs esterified in a variety of tissues of the rat were found to be approximately one-third to one-fourth the levels of F_2-IsoPs. However, in contrast to F_2-IsoPs, D_2/E_2-IsoPs cannot be detected in the circulation of humans or rats under normal circumstances, although they can be detected in the circulation of rats that have been administered CCl_4 to induce severe lipid peroxidation. The reason for this is not clear but may be due to differences in the rate of the metabolic clearance of D_2/E_2-IsoPs and F_2-IsoPs.

B. Isothromboxanes

It has previously been shown that the cyclooxygenase-derived endoperoxide, PGH_2, can also rearrange nonenzymatically to form small quantities of thromboxane A_2, a process that can be catalyzed by iron-containing porphyrin compounds [24]. Therefore, we examined whether the IsoP endoperoxides may also rearrange to form isothromboxane compounds in vivo. We have previously provided convincing evidence that this does occur [25]. Using an assay we had developed for quantification of cyclooxygenase-derived thromboxane B_2, we were able to detect significant amounts of isothromboxanes derived from the free-radical-catalyzed peroxidation of arachidonic acid both in vitro and in vivo. Structural identification of these compounds was confirmed employing various chemical and mass spectrometric approaches. Levels of isothromboxanes esterified to tissue lipids (e.g., in liver) are similar to the levels of D_2/E_2-IsoPs.

C. A_2/J_2-Isoprostanes

There has been a significant amount of interest over the past several years in a group of highly reactive PGs, termed cyclopentenone PGs, formed as dehydration products of PGD_2 and PGE_2. In aqueous or protein-containing solutions, PGE_2 readily loses a molecule of water to form PGA_2, whereas PGD_2 dehydrates to form either PGJ_2 or Δ^{12}-PGJ_2 [26]. The cyclopentenone PGs have been shown to possess significant bioactivity primarily as modulators of cellular proliferation and differentiation [27,28]. This property may, in part, be attributed to their lipophilicity in that when applied exogenously to cells in culture, cyclopentenone PGs are readily taken up by various cell lines and can be detected in nuclear fractions [29]. In addition, they rapidly adduct various important biomolecules [30]. For example, Δ^{12}-PGJ_2 forms a covalent adduct with glutathione and has

been postulated to alter cellular function by affecting redox status. Therefore, because of the interest in cyclopentenone PGs, we sought to determine whether analogous PGA_2-like and PGJ_2-like compounds, termed cyclopentenone IsoPs, might be formed via the IsoP pathway. If we were able to detect these compounds in vivo, we reasoned they might modulate certain pathophysiological responses to oxidant stress owing to their marked reactivity.

Initially, we sought to determine whether we could detect cyclopentenone IsoPs from the oxidation of arachidonic acid in vitro. For these studies, arachidonic acid was oxidized using Fe/ADP/ascorbate, as previously described [25]. Cyclopentenone compounds were then purified by Sep-Pak solid-phase extraction and thin-layer chromatography (TLC) using modifications of the methods described for the purification of PGD_2 and PGE_2 [31]. Compounds were quantified by a stable isotope dilution mass spectrometric assay using $[^2H_4]$-PGA_2 as an internal standard. Putative A_2/J_2-IsoPs were analyzed as *O*-methyloxime pentafluorobenzyl ester trimethylsilyl ether derivatives by GC/negative ion chemical ionization MS with selected ion monitoring of m/z 434 for cyclopentenone IsoPs and m/z 438 for the deuteriated internal standard. Oxidation of arachidonic acid in vitro resulted in the formation of multiple m/z 434 peaks that eluted from the GC column over approximately a 1-min interval with a retention time similar to $[^2H_4]$-PGA_2. The total amount of these compounds formed was approximately 530 ng/mg arachidonic acid. Subsequent analysis of these compounds as $[^2H_3]$-methyloxime derivatives, as $[^2H_9]$-trimethylsilyl ether derivatives, and following catalytic hydrogenation indicated that all of these compounds had one carbonyl group, one hydroxyl group, and three double bonds, as would be expected for PGA_2-like and PGJ_2-like compounds. In addition, formation of piperidyl-enol-trimethylsilyl ether derivatives confirmed the presence of PGA_2-like compounds [32]. Collectively, these data provided convincing evidence that cyclopentenone IsoPs are generated as products of free-radical-induced peroxidation of arachidonic acid in vitro.

Subsequently, studies were undertaken to determine whether these compounds were formed in vivo. A well-characterized animal model of lipid peroxidation was utilized for these experiments involving the orogastric administration of CCl_4 to rats [13]. The animals were then sacrificed, livers removed, and free cyclopentenone IsoPs measured as piperidyl-enol-trimethylsilyl ether derivatives following lipid extraction and alkaline hydrolysis of tissue lipids. Interestingly, levels of cyclopentenone IsoPs were detectable in control animals not treated with CCl_4 at levels of approximately 5 ng/g liver tissue. Treatment with CCl_4 increased these levels 24-fold. Formation of deuteriated derivatives and catalytic hydrogenation confirmed that these compounds were cyclopentenone IsoPs derived from the free-radical-catalyzed peroxidation of arachidonic acid. Thus, these studies have now provided evidence for the formation of a fourth series of PG-like

compounds derived from the free-radical-catalyzed peroxidation of arachidonic acid in vivo.

V. IMPORTANCE OF THE DISCOVERY OF ISOPROSTANES

A. Analytical Ramifications

The discovery of IsoPs is important for several reasons. First, the finding that IsoPs, and in particular F_2-IsoPs, can be generated in biological fluids in vitro has potentially important analytical ramifications for the analysis of prostanoids [6]. This applies to both physical and immunological methods of analysis. Precautions, such as the storage of fluids at −70°C or the addition of antioxidants to extraction solutions, must be taken to avoid generation of IsoPs in lipid-containing biological fluids prior to analysis [18]. F_2-IsoPs have chromatographic properties on TLC, HPLC, and GC similar to those of $PGF_{2\alpha}$ and, thus, can confound an interpretation of whether a PGF_2 compound measured by physical methods (e.g., GC/MS) is enzymatically or nonenzymatically generated [6]. Furthermore antibodies used in immunoassays for cyclooxygenase-derived PGF_2 compounds may potentially cross-react with F_2-IsoPs. For example, we have found that an antibody obtained commercially (Amersham Life Science, Arlington Heights, IL) to the PGD_2 metabolite, 9α,11β-PGF_2, exhibits significant cross-reactivity with the complex mixture of F_2-IsoPs, even though the prostane ring hydroxyls in F_2-IsoPs are oriented cis [6].

B. The Isoprostanes as an Index of Endogenous Lipid Peroxidation

A second important aspect of the discovery of IsoPs relates to the use of the measurement of IsoPs as an index of lipid peroxidation or oxidant stress in vivo. It has been recognized previously that one of the greatest needs in the field of free-radical research is the availability of a reliable noninvasive method to assess oxidative stress status in vivo in humans. This is the case because most methods available to assess oxidant stress in vivo have suffered previously from a lack of specificity and/or sensitivity or are unreliable [5]. However, evidence has been obtained that indicates measurement of IsoPs (i.e., in urine or plasma) provides a reliable noninvasive approach to assess lipid peroxidation in vivo and, thus, represents what appears to be a major advance in our ability to assess oxidative stress status in humans. Furthermore, the sensitivity of the mass spectrometric method of analysis appears sufficient to quantify levels of F_2-IsoPs in small biop-

sies of human tissue [e.g., gastric biopsies (unpublished data)], which should permit an assessment of oxidant injury in key tissues of interest.

The ability to quantify F_2-IsoPs, therefore, will potentially allow for exploration of the role of free radicals in the pathophysiology of a wide range of human diseases. It also provides an extremely valuable tool to define the clinical pharmacology of antioxidant agents. There are trials either planned or underway examining the effect of antioxidants such as vitamin C, vitamin E, or others to prevent or ameliorate some of the pathology of diseases in which free radicals have been implicated. However, such studies are hampered by insufficient information regarding what doses and combinations of antioxidants are maximally effective. Measurement of IsoPs should provide a valuable approach to define the clinical pharmacology of antioxidants. In this respect, we have previously shown that the formation of F_2-IsoPs increases significantly in animals deficient in vitamin E and/or selenium [13,33]. In addition, administration of antioxidants has been shown to inhibit the formation of IsoPs in animal models of oxidant injury [34]. More recently, we have found that the administration of a combination of antioxidants at high doses (4 g/day vitamin C, 3200 IU/day vitamin E, and 300 mg/day β-carotene) to normal volunteers for a period of 2 weeks inhibited the formation of F_2-IsoPs esterified to plasma lipids by a mean 37% (unpublished data). Furthermore, we have also examined the effect of administering 400 IU and 800 IU/day of vitamin E (α-tocopherol) for 2 weeks on plasma concentrations of unesterified F_2-IsoPs. In the group given 400 IU/day vitamin E, circulating concentrations of F_2-IsoPs decreased 25%, whereas in the group taking 800 IU/day of vitamin E, plasma levels of F_2-IsoPs fell by 37%. The lower dose approached statistical significance, whereas the higher dose produced a highly significant decrease in IsoP formation ($p = 0.007$) (unpublished data). More recently, we assessed the effect of administration of 200 mg of *d*-α-tocopherol alone, 500 mg of vitamin C alone, and the two agents in combination in a cohort of 100 men enrolled in the "Antioxidant Supplementation in Atherosclerosis Prevention" (ASAP) trial (unpublished data). After one year of treatment, plasma concentrations of F_2-IsoPs were measured to determine the effect of treatment regimens on endogenous lipid peroxidation. Vitamin E administration was found to significantly reduce plasma concentrations of F_2-IsoPs by 21% ($p = 0.003$). Vitamin C supplementation alone had no effect. In addition, in the group of men administered both agents, levels of F_2-IsoPs were not suppressed further with the addition of vitamin C to vitamin E and were actually higher with both agents compared to vitamin E alone. The reasons for this latter observation are unclear. Nonetheless, these data suggest that measurement of IsoPs can be used to quantitatively define the effects of antioxidants to inhibit free-radical processes in vivo in humans. Such information is very important to long-term studies aimed at exploring the efficacy of antioxidants to prevent free-radical-mediated pathological tissue changes associated with particular diseases.

Levels of IsoPs in normal human plasma and urine exceed levels of cyclooxygenase-derived PGs and thromboxane by more than an order of magnitude, suggesting that the formation of IsoPs is a major pathway of arachidonic acid disposition [13]. Furthermore, it is important to consider the relevance of the finding that levels of F_2-IsoPs are sufficient to be detected in every normal biological fluid that has been assayed; this includes plasma, urine, cerebrospinal fluid, bile, and gastric juice. Previously, using other methods to assess lipid peroxidation, there had been little definitive evidence indicating lipid peroxidation occurs in vivo except under abnormal circumstances of marked oxidative stress. However, the finding of detectable levels of F_2-IsoPs in all normal animal and human biological fluids and esterified in normal animal tissues indicates that there is ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in normal individuals. This finding may lend support to the hypothesis that the normal aging process is due to enhanced oxidant damage of important biological molecules over time [4]. In this regard, it has been reported that there is a trend for the formation of F_2-IsoPs to increase with age in humans [35].

C. The Isoprostanes as Mediators of Oxidant Stress

Another important aspect of the discovery of IsoPs has been the finding that several IsoPs, 15-E_2-IsoP (8-iso-PGE_2), 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$), and 15-F_{2c}-IsoP (12-epi-$PGF_{2\alpha}$) exert potent biological activity [13,23,36]. Thus, these compounds may not simply be markers of lipid peroxidation but may also participate as mediators of oxidant injury. The possibility that additional IsoPs will also be found to possess biological activity awaits the availability of additional synthetic compounds. The chemical synthesis of additional IsoPs is currently being undertaken by several laboratories. Regarding the biological activity of isothromboxanes, one might anticipate that, analogous to cyclooxygenase-derived thromboxane, only the compounds with a thromboxane A_2-like ring, but not a thromboxane B_2-like ring structure, would be bioactive. Unfortunately, because of the marked instability of the thromboxane A_2 ring, it will be difficult, if not impossible, to synthesize isothromboxane A_2 compounds for purposes of biological testing.

VI. METHOD OF ANALYSIS OF THE ISOPROSTANES

The method that we have utilized for measurement of F_2-IsoPs is a GC/negative ion chemical ionization MS assay [13,18]. It is highly sensitive, with a

lower limit of detection in the low-picogram range. Furthermore, it is highly accurate (precision = ±6%, accuracy = 96%). Previously, we have used either $[^2H_7]9\alpha,11\beta$-PGF_2 synthesized in our laboratory or commercially available $[^2H_4]PGF_{2\alpha}$ as an internal standard, but recently $[^2H_4]$ 8-iso-$PGF_{2\alpha}$, one of the more abundant F_2-IsoPs produced in vivo [37], has become available commercially. Measurement of esterified levels of F_2-IsoPs in tissues is accomplished by measurement of free compounds following alkaline hydrolysis of a lipid extract of tissue [18]. IsoPs are analyzed following conversion to pentafluorobenzyl ester trimethylsilyether derivatives. For quantification purposes, we quantify the asterisked (*) peak shown in the m/z 569 chromatogram in Fig. 1. We have previously shown that 15-F_{2t}-IsoP comprises a significant proportion of the F_2-IsoPs represented by this chromatographic peak [37].

Although highly accurate, the mass spectrometric method of assay is labor intensive and the technology is not widely available. However, both commercial enterprises and academic investigators have developed or are developing immunoassays for specific F_2-IsoPs [38], which should expand research in this area. Currently, three immunoassay kits are commercially available. Furthermore, a method was recently reported for measurement of 15-F_{2t}-IsoP using an immunoaffinity column for purification coupled with quantitation by mass spectrometry [39].

VII. QUANTIFICATION OF ISOPROSTANES AS AN INDEX OF OXIDANT STRESS

A. In Vitro Studies

A number of studies have been carried out involving the quantification of F_2-IsoPs in in vitro systems of lipid peroxidation, and F_2-IsoP formation has been compared with other markers of lipid peroxidation. This work has demonstrated the utility of measuring these compounds as a reliable index of lipid peroxidation in vitro.

The formation of F_2-IsoP has been compared to malondialdehyde (MDA) in Fe/ADP/ascorbate induced peroxidation of rat liver microsomes [40]. MDA is one of the most commonly used measures of lipid peroxidation and was quantified in these studies by measuring thiobarbituric-acid-reacting substances. Both F_2-IsoP and MDA formation increased in parallel in a time-dependent manner and correlated with the loss of arachidonic acid and with increasing oxygen concentrations up to 21%. Although the formation of F_2-IsoP correlated with other measures of lipid peroxidation in this in vitro model, as discussed later, measure-

ment of F_2-IsoPs is superior to measurements of MDA as an index of lipid peroxidation in vivo.

We and others have carried out studies examining the formation of F_2-IsoP in low-density lipoproteins (LDL) exposed to various oxidizing conditions in vitro. Much of the interest in examining this stems from the hypothesis that oxidation of LDL in vivo converts it to an atherogenic form which is taken up by macrophages in the vessel wall. Subsequent activation of these cells may play an important role in the development and progression of atherosclerotic lesions in humans [41]. Thus, we have performed studies examining the formation of F_2-IsoP in LDL that is oxidized to determine whether measurement of F_2-IsoP esterified to lipoproteins may provide an approach to assess lipoprotein oxidation in vivo [16]. These studies are also of interest because the F_2-IsoP, 15-F_{2t}-IsoP, is a vasoconstrictor and induces mitogenesis in vascular smooth-muscle cells [13] and these effects may be of relevance to the pathophysiology associated with atherosclerosis. In these studies, either plasma lipids or purified LDL from humans was peroxidized with Cu^{2+} or the water-soluble oxidizing agent 2,2-azo-bis(2-amidinopropane) (AAPH) [16]. The formation of F_2-IsoPs was compared to other markers of lipid peroxidation, including formation of cholesterol ester hydroperoxides, phospholipid hydroperoxides, loss of antioxidants, and changes in the electrophoretic mobility of LDL. In plasma oxidized with AAPH, increases in the formation of F_2-IsoP paralleled increases in lipid hydroperoxide formation and occurred only after depletion of the antioxidants ascorbate and ubiquinol-10. In purified LDL that was oxidized, formation of F_2-IsoP again correlated with increases in lipid hydroperoxides and increases in the electrophoretic mobility of LDL. Furthermore, increased F_2-IsoP formation occurred only after depletion of the antioxidants α-tocopherol and ubiquinol-10. Similar findings have been reported by Gopual and colleagues when LDL is oxidized in the presence of endothelial cells or Cu^{2+} [42]. Furthermore, FitzGerald and colleagues have reported large increases in 15-F_{2t}-IsoP in LDL oxidized in vitro in the presence of macrophages stimulated with zymosan [43]. The reason for this enhanced formation of IsoP is likely due to activation of superoxide production.

There has been significant interest in the role that the macrophage 15-lipoxygenase enzyme might play in the oxidation of lipoproteins in the vascular wall and the relation to atherosclerosis [44]. In support of a role for this enzyme in the oxidation of LDL in vivo, it has been shown that 15-F_{2t}-IsoP formation in LDL incubated with stimulated macrophages isolated from mice genetically engineered with a targeted disruption of the 15-lipoxygenase gene was significantly less than when LDL was incubated with macrophages isolated from control animals [45].

There has also been interest in the potential role of the oxidant peroxynitrite in LDL oxidation. Peroxynitrite is the coupling product of nitric oxide and superoxide. We examined the formation of F_2-IsoPs in LDL exposed to peroxynitrite

and found that peroxynitrite catalyzes the formation of F_2-IsoPs in a concentration-dependent fashion which correlated with increases in the electrophoretic mobility of LDL [46].

Taken together, these studies suggest that quantification of F_2-IsoP esterified to lipoproteins may provide a useful approach to assessing oxidation of LDL in vivo.

B. F_2-Isoprostane Quantification in Animal Models of Oxidant Stress

Evidence that measurement of IsoPs provides a valuable approach to assess oxidative stress status in vivo emerged from early studies that we carried out related to the discovery of these compounds [13,19]. Importantly, as mentioned previously, we can detect measurable levels of IsoPs in virtually every animal and human biological fluid and tissues that have been analyzed. This allows the definition of a normal range, and even small increases in IsoP formation can be quantified. Furthermore, overproduction of IsoPs has been well documented to occur in settings of oxidant injury. Initial work in vivo with the IsoPs employed two models of liver injury in rats in which lipid peroxidation had been implicated as an important factor: administration of CCl_4 to normal rats and diquat to selenium (Se)-deficient rats.

1. CCl_4-Induced Lipid Peroxidation

Administration of hepatotoxic doses of CCl_4 to rats caused hepatic lipid-esterified IsoPs to increase 200-fold within 1 h, with a subsequent decline over 24 h [19,47]. Plasma-free and lipid-esterified IsoP concentrations increased after liver levels and peaked at 4–8 h after CCl_4 administration [19]. Elevated IsoP levels were also documented in the bile [48]. Increased formation of F_2-IsoPs is proportional to the CCl_4 dose administered [47]. Furthermore, animals administered agents such as isoniazid or phenobarbital which induce hepatic cytochrome P450 enzymes and increase CCl_4 metabolism have IsoP levels higher than animals administered only CCl_4 [19]. In addition, depletion of endogenous glutathione stores markedly increases F_2-IsoP levels after the administration of CCl_4 [19]. On the other hand, circulating and tissue levels of F_2-IsoP can be decreased compared to animals administered CCl_4 alone by pretreatment of rats with the antioxidant lazaroid U78517 or cytochrome P450 inhibitors such as 4-methylpyrazole or SKF525A [19,34].

Studies carried out utilizing CCl_4 to induce oxidant injury in the rat have also illustrated that quantification of F_2-IsoP provides a much more sensitive and

accurate method to assess lipid peroxidation in vivo compared to other markers. As an example, following administration of CCl_4 to rats, levels of F_2-IsoPs esterified to lipids increased greater than 80-fold, whereas levels of MDA in the liver increased only 2.7-fold [40]. In another study, we also found that measuring F_2-IsoP afforded a more sensitive indicator of CCl_4-induced lipid peroxidation compared with measurement of lipid hydroperoxides by mass spectrometry [34].

2. Diquat-Induced Hepatic and Renal Toxicity

Diquat is a dipyridyl herbicide that undergoes redox cycling in vivo, generating large amounts of the superoxide anion and reductive mobiliziation of ferritin-bound iron. This compound causes hepatic and renal injury in rats and this effect is markedly augmented in animals deficient in Se, a trace element that is required for the enzymatic activities of glutathione peroxidase and other antioxidant proteins [49]. Previous studies have suggested that lipid peroxidation might be involved in the tissue damage associated with this agent. To study whether F_2-IsoPs were generated in increased amounts in association with diquat administration to Se-deficient animals, levels of F_2-IsoPs were quantified in plasma and tissues from Se-deficient rats following diquat administration. Selenium-deficient rats administered diquat showed 10-fold to 200-fold increases in plasma F_2-IsoPs, and the source of the IsoPs were determined primarily to be the kidney and liver [47]. Additional studies have also shown that GSH depletion increases IsoP levels significantly after the administration of diquat to rats [50].

3. Nutritional Antioxidant Deficiency

We have carried out a large number of studies examining the role of the antioxidant micronutrients vitamin E and Se in IsoP formation. Rats raised on a diet deficient in both Se and vitamin E from weaning begin to lose weight and frequently die of massive hepatic necrosis [51]. In vitro studies demonstrating that vitamin E blocks propagation of lipid peroxidation suggested that uncontrolled lipid peroxidation might be responsible for the liver injury seen in vitamin E/Se-deficient animals, although clear-cut data supporting this hypothesis were scant [52]. In an effort to examine the role of oxidant injury in combined vitamin E/Se deficiency, we quantified F_2-IsoP in plasma and tissues of deficient rats without any exogenous oxidant stress. Interestingly, plasma F_2-IsoPs in rats raised on a doubly deficient diet were sixfold higher than in rats raised on a control diet [33]. In addition, there were significant increases in phospholipid esterified F_2-IsoP levels in the tissues of deficient animals, including the liver, lung, kidney,

heart, and skeletal muscle. During this study, several animals maintained on a deficient diet developed spontaneous fulminant hepatic necrosis. Plasma and tissue IsoPs quantified in these animals just before death showed massive increases in IsoP levels, up to several hundred-fold, supporting the contention that lipid peroxidation is increased in animals deficient in both vitamin E and Se [47].

In additional studies, we have also found markedly increased baseline levels of isoprostanes both in plasma and tissues of animals deficient in vitamin E alone [33]. On the other hand, animals deficient in Se alone do not have significantly increased F_2-IsoP levels in tissues or plasma compared to Se-replete animals, unless they are exposed to an oxidant stress [33].

4. F_2-Isoprostane Levels in Other Animal Models of Oxidant Injury

A role for free radicals and lipid peroxidation in alcoholic liver damage has been controversial for many years. Previously, Nanji and colleagues reported increased plasma and lipid isoprostanes in rats fed ethanol chronically [53]. In a separate study, cimetidine given to rats to inhibit ethanol metabolism prevented the increase in F_2-IsoP formation and also prevented ethanol-induced liver injury [54].

In rats rendered Cu-deficient by reduction of dietary Cu, Cu/Zn superoxide dismutase activity is markedly reduced. In these animals, we found significantly increased levels of F_2-IsoPs esterified in plasma lipoproteins (mean 2.5-fold increase) compared to normal control animals [55]. In addition, there was a strong correlation between increased IsoP levels and vascular dysfunction. These data suggest a role for superoxide and its coupling product formed with nitric oxide, peroxynitrite, in lipoprotein oxidation, and vascular function in vivo.

Organophosphate poisoning is associated with muscle endplate necrosis and increased levels of IsoPs esterified to muscle tissue has been demonstrated in animals poisoned with organophosphates [56]. Furthermore, administration of a lazaroid antioxidant suppressed both levels of IsoPs and protected against organophosphate-induced muscle necrosis, suggesting that free radicals are involved in the pathologic changes that occur in the muscle in association with organophosphate poisoning.

Increased formation of IsoPs has also been demonstrated in settings of ischemia/reperfusion injury to both the liver and kidney [57]. Furthermore, dietary iron overload has been shown to be associated with increased levels of F_2-IsoPs esterified to lipids in the livers of rats [58]. The anesthetic halothane can induce liver injury, especially under hypoxic conditions, which is thought to involve the production of free radicals via the reductive metabolism of halothane [59]. We have demonstrated that in rats given halothane, even under normoxic

conditions, increased levels of F_2-IsoPs are present, esterified to hepatic lipids, indicative of free-radical-induced peroxidation of hepatic lipids [60].

C. Quantification of F_2-Isoprostanes to Assess the Role of Oxidant Injury in Human Diseases

From the above examples, measurement of IsoPs appears to be a reliable index of lipid peroxidation in vivo and, thus, potentially provides us with a tool to assess the role of free radicals in the pathophysiology of human disease. In this regard, we have carried out a large number of studies examining the role of oxidant stress in human diseases. One example where measurement of IsoPs has provided new evidence for a role of oxidant stress in human disease is summarized below. For a more detailed discussion regarding this topic, the reader is referred to additional references cited [61,62].

1. Hepatorenal Syndrome and Acetaminophen Poisoning

We have explored the hypothesis that oxidant stress may play a role in the hepatorenal syndrome. Hepatorenal syndrome is defined as the onset of renal failure of unknown etiology in patients with severe liver disease. The pathophysiology of the renal failure may be due to intense renal vasoconstriction, but the cause of the renal vasoconstriction remains poorly understood [63]. These patients often exhibit chronic endotoxemia and tissue hypoxia, an environment conducive for the generation of free radicals. Therefore, we quantified circulating levels of F_2-IsoPs in 12 patients with the hepatorenal syndrome and appropriate control groups including normal volunteers, patients with chronic renal failure and normal liver function, and patients with severe and mild liver disease in whom renal function was normal [64]. Circulating plasma concentrations of F_2-IsoPs were selectively increased a mean of 7.8-fold in patients with hepatorenal syndrome compared to the control groups ($p < 0.001$). We also measured circulating concentrations of F_2-IsoPs in 10 patients with acute liver and renal failure associated with acetaminophen (paracetamol) overdose. In this group, plasma F_2-IsoP concentrations were increased a mean of 9.1-fold above normal ($p < 0.001$). Whether the renal failure in these patients is due to direct nephrotoxicity from acetaminophen metabolites [65] and/or a variant of the "conventional" form of hepatorenal syndrome is not known. Furthermore, in three of these patients, we examined the effect of infusing a single dose of superoxide dismutase on circulating levels of F_2-IsoPs. In all three patients, plasma levels decreased to ~50% of preinfusion levels between 30 and 60 min after administration of the agent, consistent with an oxygen-centered radical process. Because

the duration of the effect of the administration of a single dose of superoxide dismutase is short due to its rapid elimination, the effect of the reduction of plasma concentrations of F_2-isoprostanes on renal function could not be assessed in this acute study.

These findings suggest a role for free radicals in the pathogenesis of this almost uniformly fatal disease. It is noteworthy to mention that liver transplantation in these patients is often associated with a return of normal renal function [66]. Unfortunately, many patients with hepatorenal syndrome die before a donor liver can be found. However, our results form a rational basis to explore whether antioxidant therapy may be effective in preventing death during the interval between the onset of hepatorenal syndrome and identification of a suitable donor liver. As previously discussed, both 15-F_{2t}-IsoP and 15-E_{2t}-IsoP are potent renal vasoconstrictors. Although it remains to be proven, it is attractive to consider the possibility that these IsoPs are contributing to the renal vasoconstriction that characterizes this disorder. In addition, the finding that 15-F_{2t}-IsoP induces endothelin-1 release may help to explain the large increases in plasma endothelin-1 concentrations in the hepatorenal syndrome [67]. We have previously reported that the vasoconstriction caused by 15-E_{2t}-IsoP and 15-F_{2t}-IsoP can be diminished by at least some thromboxane receptor antagonists, and data suggest that these compounds induce vascular effects by interaction with the thromboxane receptor or a similar receptor [61]. In this regard, we have a study currently underway to assess whether treatment of patients with hepatorenal syndrome with a thromboxane receptor anatagonist is associated with an improvement in renal function; preliminary results from this study appear promising.

VIII. SUMMARY

The discovery of IsoPs as products of nonenzymatic lipid peroxidation has opened up new areas of investigation regarding the role of free radicals in human physiology and pathophysiology. The quantification of IsoPs as markers of oxidative stress status appears to be an important advance in our ability to explore the role of free radicals in the pathogenesis of human disease. An important need in the field of free-radical medicine is information regarding the clinical pharmacology of antioxidant agents. Because of the evidence implicating free radicals in the pathogenesis of a number of human diseases, large clinical trials are planned or underway to assess whether antioxidants can either prevent the development or ameliorate the pathology of certain human disorders. However, data regarding the most effective doses and combination of antioxidant agents to use in these clinical trials is lacking. As mentioned previously, administration of antioxidants suppresses the formation of IsoPs, even in normal individuals. Thus, measure-

ment of IsoPs may provide a valuable approach to define the clinical pharmacology of antioxidants.

In addition to being markers of oxidative stress, several IsoPs possess potent biological activity. The availability of additional IsoPs in synthetic form should broaden our knowledge concerning the role of these molecules as mediators of oxidant stress.

Despite the fact that considerable information has been obtained since the initial report of the discovery of IsoPs [6], much remains to be understood about these molecules. With continued research in this area, we believe that much new information will emerge that will open up additional important new areas for future investigation.

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